

# Uni-site catalysis by *Escherichia coli* $F_1$ -ATPase with different numbers of bound nucleotides

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We prepared two types of *E. coli*  $F_1$  by slightly different gel filtration procedures of the purified  $F_1$ :  $F_1$ (II) contained about 2 mol, and  $F_1$ (V) about 5 mol of bound adenine nucleotides per mol of the enzyme. Thus  $F_1$ (II) had more than 2, possibly 3, vacant catalytic sites, while  $F_1$ (V) had less than one vacant catalytic site. The rate of ATP hydrolysis in uni-site catalysis (in the presence of inorganic phosphate) was about 3-fold higher with  $F_1$ (II) than with  $F_1$ (V), suggesting that ADP and inorganic phosphate bound at the catalytic sites of  $F_1$ (V) changed the kinetics of uni-site catalysis significantly.

ATPase,  $H^+$ ;  $F_1$ ; Uni-site catalysis; Bound nucleotide; (*Escherichia coli*)

## 1. INTRODUCTION

The  $H^+$ -ATPase of oxidative phosphorylation is formed from a catalytic sector  $F_1$  ( $F_1$ -ATPase) and a  $H^+$  pathway  $F_0$  (for reviews, see [1–4]). Six nucleotide-binding sites have been found in  $F_1$  from bovine mitochondria [5,6] and *E. coli* [7,8], and classified as 3 catalytic (exchangeable) and 3 non-catalytic (non-exchangeable) sites according to their abilities to exchange bound nucleotide rapidly during multi-site (steady-state) catalysis in the presence of excess ATP. Mitochondrial  $F_1$  preparations with different numbers of bound nucleotides have been prepared [9–11]. Kinetic studies on ATP hydrolysis by  $F_1$  indicated the presence of positive cooperativity in multi-site catalysis. ATP is hydrolyzed only slowly in uni-site (single site or non-steady-state) catalysis in which the molar ratio of ATP to  $F_1$  is less than 1, but the rate of ATP hydrolysis becomes  $10^4$ – $10^6$ -fold faster on addition of excess ATP (multi-site catalysis) [12,13]. Thus the rate of ATP hydrolysis and release of ADP and phosphate from the first catalytic site is accelerated by the binding of ATP to the second and third catalytic sites. However, the effects of binding of nucleotides to the catalytic or non-catalytic sites on uni-site catalysis have not been reported.

In this study we prepared two types of *E. coli*  $F_1$ ,  $F_1$ (II) and  $F_1$ (V) containing 2 and 5 mol, respectively,

of nucleotides bound per mol of  $F_1$ , and found that uni-site catalysis was significantly faster with  $F_1$ (II) than with  $F_1$ (V).

## 2. EXPERIMENTAL PROCEDURES

$F_1$  was purified from *E. coli* strain KY7485 as described [14], and stored at  $-80^\circ\text{C}$  in 50 mM Tris-Cl (pH 8.0) containing 1.0 mM ATP, 0.5 mM EDTA/Na and 10% glycerol. Samples of  $F_1$  with defined numbers of bound nucleotides ( $F_1$ (II) and  $F_1$ (V)) were prepared at 22–25°C by passing the stored  $F_1$  (1–4 mg/ml) through a centrifuge column [15] (Sephadex G-50 fine,  $0.5 \times 5.5$  cm) equilibrated with TMP buffer (50 mM Tris- $\text{SO}_4$  (pH 8.0) containing 0.5 mM  $\text{MgSO}_4$  and 1 mM  $\text{K}_2\text{HPO}_4$ ) and TEP buffer (50 mM Tris- $\text{SO}_4$ , pH 8.0, containing 0.5 mM EDTA/Na and 1 mM  $\text{K}_2\text{HPO}_4$ ). The same buffers without 1.0 mM  $\text{K}_2\text{HPO}_4$  were also used. The two preparations of  $F_1$  had similar subunit ratios as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Coomassie brilliant blue staining) [14].

Uni-site hydrolysis of ATP was assayed as described [16]: 0.25  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP was incubated at 25–27°C in 50 mM Tris- $\text{SO}_4$ , pH 8.0, containing 1 mM  $\text{K}_2\text{HPO}_4$  and 0.5 mM  $\text{MgSO}_4$ . For measurement of bound nucleotides,  $F_1$  was incubated at 70°C for 10 min, stood on ice for 5 min, and centrifuged at  $12000 \times g$  for 10 min. The supernatant was subjected to high-performance liquid chromatography (HPLC) in a  $\text{C}_{18}$  column (MS Pack  $0.46 \times 15$  cm) in 50 mM  $\text{KH}_2\text{PO}_4$  containing 0.6 mM tetra-*N*-butylammonium bromide and 11–12% (v/v) methanol, pH 6.4 (flow rate 0.4 ml/min) and amounts of ATP and ADP were determined from the absorbances of the respective peaks at 259 nm. Protein was measured by the methods of Bradford et al. [17] (Bio-Rad protein assay reagent) and Smith et al. [18] (BCA protein assay reagent, Pierce) with bovine serum albumin (Pierce) as a standard. [ $\alpha$ - $^{32}\text{P}$ ]ATP (21 Ci/mmol) and [ $\gamma$ - $^{32}\text{P}$ ]ATP (9 Ci/mmol) were purchased from DuPont/NEN Research Products. Radioactive bound nucleotides were measured in solvent-free scintillation medium (Ready Cap, Beckman). Other chemicals used were of the highest grade available commercially.

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## 3. RESULTS AND DISCUSSION

Kironde and Cross [9] reported that bovine  $F_1$  stored as an ammonium sulfate precipitate contained 3 mol of adenine nucleotides per mol enzyme (2 at non-catalytic sites and 1 at a catalytic site) after its gel filtration through a centrifuge column.  $F_1$  containing 5 mol of adenine nucleotides was obtained by incubating this  $F_1$  with excess MgATP. Similarly, *E. coli*  $F_1$  containing about 5 mol of ADP ( $F_1(V)$ ) could be obtained by passing the  $F_1$  (stored frozen in solution containing 1 mM ATP and 0.5 mM EDTA/Na) through a centrifuge column equilibrated with TMP buffer (table 1).  $F_1$  containing only two adenine nucleotides (about 1 mol each of ADP and ATP) was obtained by passing the stored  $F_1$  through two successive centrifuge columns in TEP buffer ( $F_1(II)^*$ ). The bound ATP in  $F_1(II)^*$  was hydrolyzed to ADP in a centrifuge column equilibrated with TMP buffer (table 1,  $F_1(II)$ ).  $F_1(II)$  could bind 3 more mol of ADP incubated with 2 mM ATP and 0.5 mM  $MgSO_4$ .

On incubation of  $F_1(II)$  with  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ ,  $F_1(V)$  with 2 unlabeled and 3 radioactive adenine nucleotides was obtained. When this radioactive  $F_1$  was incubated with 2 mM ATP and 0.5 mM  $MgSO_4$ , the 2 radioactive nucleotides were rapidly exchanged with unlabeled nucleotides (fig.1), although the total number of bound nucleotides, estimated by HPLC did not change. The radioactive nucleotides were not exchanged during incubation without ATP. These results suggest that two radioactive adenine nucleotides bound to the catalytic sites were rapidly replaced by unlabeled nucleotides during multi-site catalysis of the enzyme. The other nucleotides (two unlabeled and one radioactive) may be at non-catalytic sites. The radioactive adenine nucleotide remaining in  $F_1$  was released from the non-

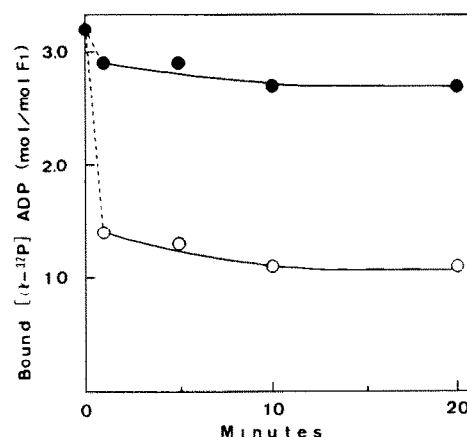


Fig.1. Release of radioactive adenine nucleotides from catalytic and non-catalytic sites of  $F_1(V)$ .  $F_1(V)$ , containing two unlabeled and 3 radioactive adenine nucleotides, was incubated in 50 mM Tris- $SO_4$ , pH 8.0, containing 0.5 mM  $MgSO_4$  with (○) or without (●) 2 mM ATP. At the times indicated, aliquots of the incubation mixtures were introduced into centrifuge columns equilibrated with TMP buffer, and the radioactivity in  $F_1$  was determined.

catalytic site when  $F_1$  was passed through two centrifuge columns equilibrated with TEP buffer, indicating that the nucleotide was at the non-catalytic site, as shown for bovine  $F_1$  [10]. As the radioactive  $F_1(V)$  formed from  $F_1(II)$  had 2 adenine nucleotides at the catalytic sites,  $F_1(V)$  had less than one vacant catalytic site, and  $F_1(II)$  had at least 2, possibly 3, vacant catalytic sites.  $F_1(II)$  and  $F_1(V)$  could also be prepared using centrifuge columns equilibrated with phosphate-free buffer (data not shown).

We compared the kinetics of uni-site catalysis with  $F_1(II)$  and  $F_1(V)$ , which have different numbers of va-

$F_1$	Procedure	Bound nucleotides (mol/mol $F_1$ )		
		ADP	ATP	Total
$F_1(V)$	$F_1 \rightarrow cc(TMP)$	4.9	0.0	4.9
$F_1(II)^*$	$F_1 \rightarrow cc(TEP) \times 2$	1.2	0.9	2.1
$F_1(II)$	$F_1(II)^* \rightarrow cc(TMP)$	2.3	0.0	2.3
$F_1(V)$	$F_1(II) \rightarrow cc(TMP) \rightarrow +Mg \cdot ATP \rightarrow cc(TMP)$	5.1	0.0	5.1

$F_1$  that had been stored at  $-80^\circ\text{C}$  in 50 mM Tris-Cl, pH 8.0, containing ATP, EDTA and glycerol was thawed and applied to a centrifuge column (cc) equilibrated with TMP or TEP buffer. When necessary, samples were applied to two successive centrifuge columns ( $\times 2$ ). Values are averages for multiple assays (at least 8 assays). Deviations were within 10–20%.  $F_1$ s prepared by the same procedures but with potassium phosphate-free buffers had essentially the same amounts of bound nucleotides as those prepared in the above conditions

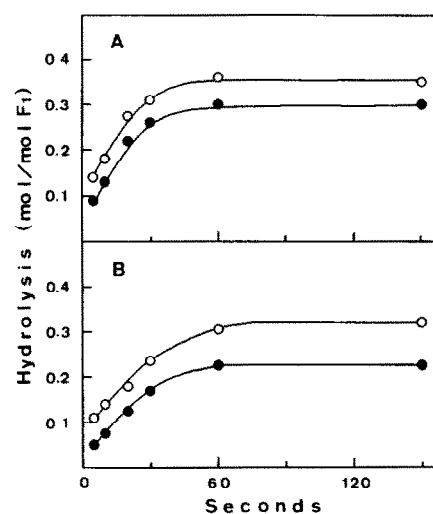


Fig.2. Uni-site hydrolysis of ATP by  $F_1(II)$  and  $F_1(V)$ . Mixtures of  $0.25 \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and  $0.5 \mu\text{M}$   $F_1(II)$  (A) or  $F_1(V)$  (B) in 50 mM Tris- $SO_4$ , pH 8.0, containing 1 mM  $K_2HPO_4$  and 0.5 mM  $MgSO_4$  were incubated for the indicated times. Reactions were terminated by addition of perchloric acid (●) (acid quench) or 1 min after addition of  $2 \times 10^4$  molar excess of non-radioactive ATP (○) (cold chase).

Table 2

Uni-site hydrolysis of ATP by  $F_1$  preparations with defined numbers of bound nucleotides

Assay condition	$F_1$	$k_1$ ( $\times 10^{-4} \text{ M}^{-1} \cdot \text{s}^{-1}$ )	Initial rate ( $\times 10^{-9} \text{ mol/mg} \cdot \text{min}$ )
+ $\text{K}_2\text{HPO}_4$	$F_1(\text{V})$	4.2	1.6
	$F_1(\text{II})$	8.3	4.7
	$F_1(\text{V})^a$	5.3	1.6
- $\text{K}_2\text{HPO}_4$	$F_1(\text{V})$	6.9	5.1
	$F_1(\text{II})$	7.7	5.3

<sup>a</sup>  $F_1(\text{V})$  obtained from  $F_1(\text{II})$  (see table 1)

$k_1$  is the rate constant of binding of ATP to  $F_1$  in uni-site hydrolysis and was calculated from the results of cold chase experiments (fig.2). The initial rate of ATP hydrolysis was determined from the amount of inorganic phosphate released after acid quench. Preparations of  $F_1$  had the numbers of nucleotides shown in table 1

cant catalytic sites. The initial rate (acid quench) of ATP hydrolysis by uni-site catalysis (determined by measuring radioactive inorganic phosphate bound to  $F_1$  and released into the medium) by  $F_1(\text{II})$  was about  $4.7 \times 10^{-9} \text{ mol/mg} \cdot \text{min}$  and was 3-fold faster than the initial rate ( $1.6 \times 10^{-9} \text{ mol/mg} \cdot \text{min}$ ) catalyzed by  $F_1(\text{V})$  (fig.2). The release of radioactive inorganic phosphate on addition of excess unlabeled ATP (cold chase) was also measured: the value of  $k_1$  (the rate of binding of ATP) of  $F_1(\text{II})$  was 1.6 ~ 2.0 times that of  $F_1(\text{V})$  (table 2). The  $F_1(\text{V})$  formed from  $F_1(\text{II})$  showed essentially the same values of  $k_1$  and initial rate of uni-site hydrolysis as  $F_1(\text{V})$  obtained directly from the stored  $F_1$  (table 2). Similarly,  $F_1(\text{II})$  formed from  $F_1(\text{V})$  showed essentially the same kinetic properties as  $F_1(\text{II})$  prepared directly from the stored  $F_1$  (data not shown). These results indicate that the low activity of  $F_1(\text{V})$  was not due to irreversible change (e.g. denaturation) of the enzyme.

The  $F_1(\text{V})$  showed a lower rate of uni-site catalysis than  $F_1(\text{II})$  when the hydrolysis was assayed in the presence of inorganic phosphate, but essentially the same values of  $k_1$  and initial rate of hydrolysis as  $F_1(\text{II})$  in the absence of inorganic phosphate (table 2,  $\text{K}_2\text{HPO}_4$ ). It should be noted that we could not obtain  $F_1$  without bound nucleotides by passing  $F_1$  through a gel filtration column with 50% glycerol, as previously reported for *E. coli*  $F_1$  [7,8,19].

The present results clearly indicate that change in the kinetics of uni-site catalysis by  $F_1$  in the presence of in-

organic phosphate depends on the preparation of  $F_1$ , possibly due to differences in the numbers of vacant catalytic sites in the  $F_1$  molecule. As described above,  $F_1(\text{V})$  had only a single vacant catalytic site, whereas  $F_1(\text{II})$  had possibly 3 vacant catalytic sites. Thus we conclude that the presence of ADP in the catalytic sites of  $F_1(\text{V})$  together with inorganic phosphate significantly lowered the rate of uni-site catalysis of ATP at the third catalytic site.

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